AN *IN VITRO* EFFECT OF THYROID HORMONE UPON BONE MARROW SYNTHESIS OF HEMOGLOBIN

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1. Introduction

For several years thyroid hormones have been known to exert an influence upon the production of red blood cells [1–3]. It has not been resolved however, whether the hormones acted directly upon erythroid precursors in the bone marrow or indirectly through the increased production of erythropoietin. The data of Crafts and Meineke [3] comparing the effects of thyroxine and dinitrophenol upon oxygen uptake and red cell production suggest that thyroxine probably has a direct effect upon the bone marrow in addition to that mediated by increased oxygen consumption. The present investigation demonstrates that L-thyroxine has an *in vitro* effect upon the synthesis of hemoglobin by red blood cell precursors in the bone marrow.

2. Materials and methods

Bone marrow was obtained from normal New Zealand white rabbits and animals made anemic by the injection of acetylphenylhydrazine [4]. After the rabbits were sacrificed by cardiac puncture, femora and humeri were removed and immersed in ice cold saline until the bone marrow was extracted. Epiphyses were removed and the marrow blown out of the diaphyses into the incubation medium. Marrow clumps were gently dispersed into homogeneous suspensions by passage through nylon mesh (MBCO, Macalaster Bicknell Co.). All reactions were carried out at 37° in stoppered flasks, using 3% cell suspensions in a total volume of ten ml.

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2.1. *Hemoglobin synthesis*

All incubations were carried out in commercial NCI medium (Grand Island Biological Co.). The isotopic precursors were purchased from Schwarz or New England Nuclear Corp. Reactions were terminated after 90 min by centrifuging and washing the cells in ice cold saline. Cells were disrupted by gentle homogenization in ten volumes of 1.5 mM $MgCl_2$ using 5 strokes of the tight Dounce glass homogenizer. Cell debris and nuclei were removed by centrifugation at 144,000 *g* for 90 min. The supernatant containing the hemoglobin was dialyzed for 24 hr against 0.01 M phosphate buffer, pH 6.9, containing 1 mM KCN. The dialyzate was layered on a 15 × 1.5 cm CM Sephadex C50 column. Hemoglobin was eluted using a linear gradient in 0.01 M phosphate buffer, from pH 6.9 to pH 8.5 according to the method of Winterhalter [5]. Radioactivity was determined on TCA precipitated material. Purity of the hemoglobin was tested by preparing globin by the acid acetone procedure of Anson and Mirsky [6]. After the addition of cold carrier globin obtained from mature rabbit erythrocytes, the globin was separated into alpha and beta chains [7]. The identity of the sample was further tested by acrylamide gel electrophoresis in veronal buffer at pH 8.6 against hemoglobin from mature erythrocytes acting as marker.

2.2. *Heme synthesis*

Bone marrow suspensions were incubated as described for hemoglobin synthesis. At the end of the incubations cold carrier hemoglobin was added and hemin was twice crystallized and counted as described by Grayzel et al. [8]. All incubations were performed in duplicate.

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Table 1
Effect of thyroxine on hemoglobin synthesis

Tissue	Additions	% of Control ± S.D.
Erythroid bone marrow	None	100
	L-thyroxine (10^{-7} M)	131 ± 10 (5)
Reticulocytes	None	100
	L-thyroxine (10^{-7} M)	100 ± 8 (5)

Hemoglobin was purified on CM Sephadex C-50 columns and specific activity was determined as cpm per mg of Hgb. The radioactive precursor was either ^{14}C-δ-aminolevulinic acid or ^{14}C amino acid. Numbers in parentheses are the number of separate experiments.

Reticulocytes obtained from anemic rabbits were separated from the plasma by centrifugation and washed three times with cold saline. Reticulocytes were incubated under the same conditions as described for the bone marrow.

In all studies using L-thyroxine the hormone was present from the start to the incubation at a concentration of 10^{-7} M. The hormone was obtained commercially from Calbiochem. Actinomycin D (Merck, Sharp and Dohme) was used at a concentration of 0.01 mg/ml.

3. Results and discussion

The addition of L-thyroxine to incubations of bone marrow cells from anemic rabbits stimulated the synthesis of hemoglobin an average of 31% in five different experiments (table 1). This was found to be statistically significant using Student's t, with p= 0.005. These observations are in agreement with those of Necheles [9] who reported stimulation of protein synthesis by the addition of L-thyroxine to incubations of bone marrow cells. The identity of the protein stimulated in the present experiments was confirmed as hemoglobin by acrylamide gel electrophoresis and by separation of the globin into alpha and beta chains. Assay of individual tubes of the chain column eluate showed the radioactivity following the optical density profile of the carrier globin. Both chains were labelled. It is unlikely that this stimulation was due to an effect of the hormone upon the reticulocytes present in the marrow preparations since hemoglobin synthesis by reticulocytes was unaffected by the addition of the hormone. The lack of response to the hormone by reticulocytes suggested that the increase in hemoglobin synthesis may be dependent on RNA synthesis which no longer occurs in the reticulocyte.

Table 2
Effect of thyroxine and actinomycin D on hemoglobin synthesis.

Additions	Concentration	Cpm/mg Hgb	% of Control
None		581	100
Actinomycin D	0.01 mg/ml	550	95
L-thyroxine	10^{-7} M	766	133
Actinomycin D and L-thyroxine	0.01 mg/ml 10^{-7} M	542	93

Hemoglobin was purified on CM Sephadex C-50 columns. ^{14}C-δ-amino-levulinic acid was the isotopic precursor.

Table 3
Effect of thyroxine on heme synthesis in normal bone marrow.

Additions	Concentration	Precursor	cpm ± S.E.
None	–	^{14}C-Glycine	170± 0
L-thyroxine	10^{-7} M		152± 10
None	–	^{14}C-Ala	246± 11
L-thyroxine	10^{-7} M		323± 13

Heme was twice crystalized and radioactivity determined by counting an infinite thickness. All incubations were performed in duplicate.

Actinomycin D which inhibits DNA dependent RNA synthesis [10], had no significant effect upon the base line incorporation of δ-amino-levulinic acid into purified hemoglobin. The antibiotic however, completely blocked the stimulation effected by L-thyroxine (table 2). Similar effects were observed when an amino acid was the radioactive precursor employed except that the addition of the inhibitor not only blocked the stimulation effected by the hormone but also reduced the base line synthesis of hemoglobin 28%. Table 3 shows the effect of L-thyroxine upon heme synthesis by bone marrow cells from a normal rabbit. After 90 min of incubation the hormone had no ef-

fect upon the enzyme δ-aminolevulinic acid synthetase which catalyzes the condensation of glycine and succinyl CoA to form δ-aminolevulinic acid. When this compound was the radioactive precursor however, there was a greater than 30% stimulation of heme synthesis. There was no significant difference in incorporation between duplicate samples. The stimulation of δ-aminolevulinic acid incorporation into heme, while glycine incorporation was not affected suggests globin synthesis was stimulated directly and not by means of increased heme synthesis.

In conclusion, evidence has been presented that hemoglobin synthesis by immature erythroid cells is stimulated by L-thyroxine. This stimulation is dependent on RNA synthesis and therefore presumably occurs at an early stage of erythroid development.

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